

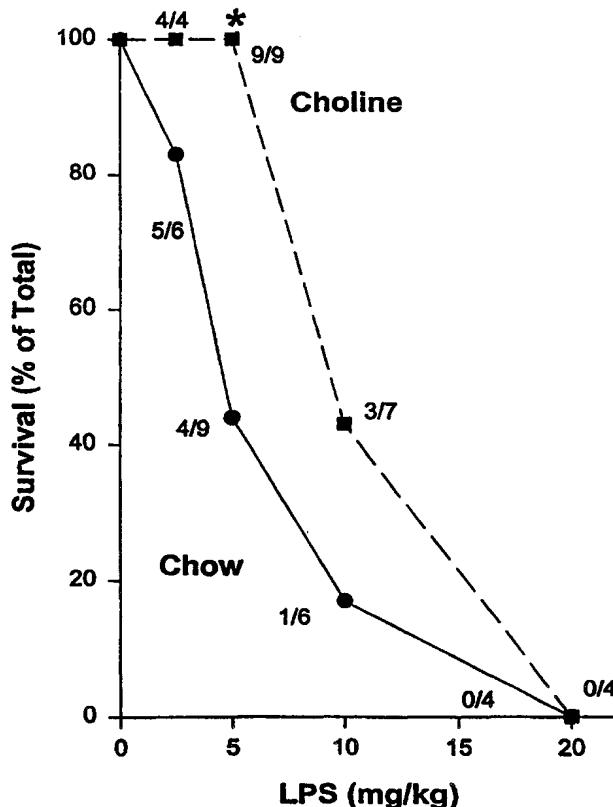
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/00		A2	(11) International Publication Number: WO 98/32428
			(43) International Publication Date: 30 July 1998 (30.07.98)
(21) International Application Number:	PCT/EP98/00373		
(22) International Filing Date:	23 January 1998 (23.01.98)		
(30) Priority Data:	08/789,773 27 January 1997 (27.01.97)	US	
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application	US 08/789,773 (CIP)		
Filed on	27 January 1997 (27.01.97)		
(71) Applicant (for all designated States except US):	NOVARTIS NUTRITION AG [CH/CH]; Monbijoustrasse 118, CH-3001 Bern (CH).		
(72) Inventors; and			
(75) Inventors/Applicants (for US only):	SCHNEIDER, Heinz [CH/CH]; Buillard, CH-1792 Cordast (CH). THURMAN, Ronald, G. [US/US]; 810 Mt. Creek Road, Chapel Hill, NC 27516 (US).		
(74) Agent:	SMOLDERS, Walter; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH).		

(54) Title: COMPOSITIONS COMPRISING CHOLINE AND USE OF CHOLINE TO TREAT ENDOTOXIC SHOCK

(57) Abstract

The invention provides a method for the treatment of endotoxic shock comprising administering to a human or other mammal in need of such a treatment an effective amount of choline for reducting endotoxin-induced injury and mortality and a nutritional composition comprising choline, whereby the composition provides, in a daily dosage form, from 1.5 to 20 g of choline.



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Compositions comprising choline and use of choline to treat endotoxic shock

The present invention relates to the reduction of endotoxin-induced injury and mortality by administration of choline

Gram-negative bacterial infection involves the release of massive amounts of endotoxin into the systemic circulation. Endotoxins (lipopolysaccharides) are a cell wall component of gram-negative bacteria and are cleared from the systemic circulation largely by Kupffer cells, the resident macrophage of the liver. Phagocytosis of endotoxin activates Kupffer cells to release prostaglandins, cytokines and free radicals that lead to hypermetabolism and cause liver injury. The release of endotoxin and Kupffer cell activation are also important events in the genesis of alcoholic liver disease.

Sepsis or endotoxic shock is a major health problem which occurs not infrequently following major surgery and trauma and often leads to refractory hypotension and multiple organ failure. There are many deaths from these conditions annually which represent a unique challenge in critical care medicine.

It has now surprisingly been found that choline reduces endotoxin-induced injury and mortality and thereby protects against endotoxic shock and its consequences.

The present examples demonstrate the effectiveness of choline in the prevention of mortality in a rat model of endotoxin shock. In addition, choline also prevented liver and lung injury. Previously, it has been shown that dietary supplementation with glycine blocks endotoxin-induced injury and subsequent mortality. In this study, feeding a diet rich in both glycine and choline was surprisingly even more effective in preventing mortality, suggesting that choline and glycine act via different mechanisms. Hence, the examples clearly demonstrate the effectiveness of choline in preventing

endotoxin-induced injury and mortality and in preventing endotoxin-related injuries such as alcoholic liver injury.

The present invention accordingly provides the use of choline in the preparation of a medicament or nutritional formulation for the reduction and/or prevention of endotoxin-induced injury and mortality.

Further the invention provides a method for the treatment of endotoxic shock comprising administering to a human or other mammal in need of such a treatment an effective amount of choline for reducing endotoxin-induced injury and mortality.

For use in the compositions, formulations, diets and methods of the invention, choline is conveniently employed as a free base, in physiologically acceptable salt form, in form of lecithin, and/or in form of natural sources rich in lecithin or choline such as egg yolk, kidney, liver, heart, seeds, vegetables, and legumes. Preferably choline is employed as choline tartrate.

An "effective amount of choline" as used herein lies typically in the range of 1.5 to 20 g, preferably 3 to 15 g, more preferred 7 to 12 g of the free base per 24 hours. The person skilled in the art will know how to adjust the dosage if other sources than the free base of choline are used. If e.g. lecithin is used the amount by weight of lecithin used will roughly be five times the amount of free base of choline. The amount of choline to be administered depends to a large extent on the patient's specific requirements. Such daily amounts of choline are suitable for treatment of the desired effects as well as for prophylactic/pretreatment.

The invention also provides for the use of a mixture of glycine and choline in the preparation of a medicament or nutritional formulation for the reduction and/or prevention of endotoxin-induced injury and mortality.

Also provided is a method for the treatment of endotoxic shock comprising administering to a human or other mammal in need of such a treatment a combined amount of choline and glycine effective for reducing endotoxin-induced injury and mortality.

For use in the compositions, formulations, diets and methods of the invention, glycine is conveniently employed in the form of the free base of the amino acid, in the form of glycine precursors, in particular alanine or serine, likewise in free amino acid base form, in physiologically acceptable salt form of said amino acids, or in form of mixtures of said amino acids and/or physiologically acceptable salts thereof. Glycine is preferably used in form of the free base of the amino acid, in physiologically acceptable salt form or in the form of a mixture of glycine in free amino acid base form with glycine in physiologically acceptable salt form; most preferably glycine is in free amino acid base form. Glycine may also be used in the form of dipeptides according to the invention.

An "effective amount of glycine" as used herein lies typically in the range of 1.5 to 75 g, preferably 2 to 60 g, particularly preferred 18 to 40 g of the free base of the amino acid per 24 hours. The person skilled in the art will know how to adjust the dosage if other sources than the free base of glycine are used. The amount glycine to be administered depends to a large extent on the patient's specific requirements. Such daily amounts of glycine are suitable for treatment of the desired effects as well as for prophylactic/pretreatment.

Typically the ratio of choline:glycine is from 1:1 to 1:50, preferably 1:1.5 to 1:10, particularly preferred from 1:2 to 1:5. The ratio given is with respect to the free base of choline and glycine the person skilled in the art will know how to adjust the ratios if other sources of choline and glycine are used.

The invention further provides the use of choline in the preparation of a medicament or nutritional formulation for reducing and/or preventing endotoxin-related injuries such as alcoholic liver injury.

Still further provided is a method for the treatment of endotoxin-related injury comprising administering to a human or other mammal in need of such a treatment an effective amount of choline to reduce the endotoxin-related injury such as alcoholic liver injury.

Further provided is a nutritional composition comprising as a daily dosage form, from 1.5 to 20 g of choline and a nutritionally acceptable carrier.

Also provided is a nutritional composition comprising:

1.5 to 20 parts of choline,

1.5 to 75 parts of glycine, and

a nutritionally acceptable carrier,

wherein in a daily dosage form, the composition provides from 1.5 to 20 g of choline and from 1.5 to 75 g of glycine.

The nutritional formulation or medicament may be administered either prophylactically, e.g. preoperatively, in the acute phase, e.g. postoperatively, or both.

The nutritional formulation or medicament may be administered to the patient enterally or parenterally. The enteral administration route is preferred, particularly for subsequent or prophylactic treatment; particularly contemplated enteral administration routes are oral administration and/or tube feeding. The medicament or formulation is conveniently administered in the form of an aqueous liquid. The medicament or formulation in a form suitable for enteral application is accordingly in aqueous or in powder form, preferably in powder form, whereby the powder is conveniently added to water prior to use. For use in tube feeding, the amount of water to be added will depend, *inter alia*, on the patient's fluid requirements and condition. It will be appreciated that, for acute treatment, the parenteral application route may be preferred.

Generally, it is indicated to use choline in combination with one or more of the following components:

- (i) glycine;
- (ii) omega-3 polyunsaturated fatty acids (PUFAs) where desired in admixture with omega-6 PUFAs;
- (iii) L-arginine or other physiologically acceptable compounds associated with the synthesis of polyamines, or mixtures thereof; and
- (iv) a nucleobase source.

Nucleobase sources suitable for use in combination with the amino acids of the invention comprise or consist of natural nucleobases, nucleosides, nucleotides, RNA, DNA, equivalents thereof and/of mixtures comprising one or more of these compounds.

Natural nucleobases include the purines adenine and guanine as well as the pyrimidines cytosine, thymine and uracil. Where the nucleobase source is in the form of free nucleobases, it is preferably uracil.

Natural nucleosides include the ribose nucleosides adenosine, guanosine, uridine and cytidine and the deoxyribose nucleosides deoxyadenosine, deoxyguanosine, deoxythymidine and deoxycytidine.

Natural nucleotides include phosphate esters of natural nucleosides, such as the monophosphates adenylylate (AMP), guanylylate (GMP), uridylylate (UMP), cytidylate (CMP), deoxythymidylate (dTMP), deoxycytidylate (dCMP), and diphosphates and triphosphates of natural nucleosides such as ADP and ATP.

A purified nucleobase source, such as yeast is preferred. However, other sources such as meat and the like may be used. Preferably the nucleobase source is RNA.

Accordingly, the invention provides medicaments or nutritional formulations comprising effective amounts of:

- (a) choline (component (a)) in association with one or more components selected from
- (b) glycine (component (b));
- (c) omega-3 PUFAs where desired in admixture with omega-6 PUFAs (component (c));
- (d) L-arginine or other physiologically acceptable compounds associated with the synthesis of polyamines, or mixtures thereof (component (d)); and
- (e) a nucleobase source (component (e)).

Said medicaments and nutritional formulations are hereinafter designated "diets of the inventions".

One unit dose of such a medicament or nutritional formulation preferably comprises 1.5 to 20 parts by weight of component (a) in association with the following amounts of one or more components selected from (b) to (e): 1.5 to 75 parts by weight of component (b), 0.1 to 20 parts by weight of component (c), 3 to 40 parts by weight of component (d) and 0.1 to 4.0 parts by weight of component (e).

Particularly preferred one unit dose comprises 1.5 to 20 parts by weight of component (a) in association with the following amounts of one or more components selected from (b) to (e): 2 to 60 parts by weight of component (b), 2 to 5 parts by weight of component (c), 7.5 to 20 parts by weight of component (d) and 1.7 to 2.0 parts by weight of component (e).

The amount of components (a) to (e) administered daily will conveniently correspond to 1.5 to 20 g for component (a), 1.5 to 75 g, preferably 2 to 60 g,

particularly preferred 18 to 40 g for component (b), 0.1 to 20 g, preferably 2 to 5 g, for component (c), 3 to 40 g, preferably 7.5 to 20 g, for component (d) and 0.1 to 4.0 g, preferably 1.7 to 2.0 g, for component (e).

With respect to component (e) the above dosage is indicated for RNA, DNA, nucleosides or nucleotides. For nucleobases one weight unit of nucleobases is regarded to be equivalent to 2.5 to 3.0 weight units of RNA, DNA, nucleosides or nucleotides.

Where medicaments or nutritional formulations comprising choline in combination with one or more of the above-mentioned components (b) to (e) are used, such medicaments or nutritional formulations will conveniently comprise in one unit dose

- (a) 1.5 to 20 parts by weight of choline,
in combination with one or more compounds selected from the group consisting of
 - (b) 1.5 to 75 parts by weight glycine
 - (c) 2 to 5 parts by weight omega-3 polyunsaturated fatty acids;
 - (d) 7.5 to 20 parts by weight L-arginine or L-ornithine, or mixtures thereof; and
 - (e) 1.7 to 2.0 parts by weight RNA.

Preferred medicaments or nutritional formulations comprise in one unit dose:

- (a) from 1.5 to 20 parts by weight of choline, in association with
- (b) 1.5 to 75 parts by weight, preferably 2 to 60 parts by weight, particularly preferred 18 to 40 parts by weight, of glycine.

Further preferred medicaments or nutritional formulations comprise in one unit dose:

- (a) from 1.5 to 20 parts by weight choline, in association with

omega-3 PUFAs which are conveniently protected against peroxidation.

Physiologically acceptable ways of protecting omega-3 PUFAs against peroxidation are known in the art. They include physiologically acceptable micro-encapsulation of omega-3 PUFAs and the use of physiologically acceptable antioxidants.

A typical example suitable for use as physiologically acceptable micro-encapsulation agents is starch. The micro-encapsulation can be effected in a manner known per se. The micro-encapsulates may be coated in a manner known per se, by physiologically acceptable coating agents such as Gum Arabic.

Typical examples of antioxidants suitable for use in the method of the invention include antioxidant vitamins such as Vitamin C, Vitamin E or mixtures thereof.

The amount of antioxidant added should be sufficient to prevent peroxidation of the omega-3 PUFAs. Such amounts can be easily calculated. In general, for convenience, any antioxidants employed to prevent peroxidation, will be employed in excess. It will be appreciated that the presence of any other agent administered in association with the omega-3 PUFAs may require adjustment of the amount of antioxidant to be employed.

The omega-3 PUFAs may be employed in a form suitable for the physiological supply of omega-3 PUFAs, e.g. in free acid form, in triglyceride form, or in the form of physiologically acceptable natural sources of omega-3 PUFAs. Such natural sources include linseed oil and fish oils such as menhaden oil, salmon oil, mackerel oil, tuna oil, cod-liver oil and anchovy oil. Said natural sources, in particular, the fish oils, comprise substantial amounts of omega-3 fatty acids. Where the omega-3 PUFAs are employed in triglyceride form, said triglycerides may comprise esters with other physiologically acceptable fatty acids. Preferred omega-3 PUFAs include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in free acid form, in

triglyceride form or in form of natural sources having a high EPA and/or DHA content.

In general, favorable effects are obtained when administering the nutritonal formulations in the form of a formula diet, which may, depending on the circumstances be a complete formula diet (i.e., a diet supplying essentially all required energy, amino acids, vitamins, minerals and trace elements) or a diet supplement. The formula diet will conveniently be taken in aqueous liquid form. A formula diet accordingly may comprise a source of carbohydrates, lipids fat (fat source) and protein (nitrogen source), and choline as a free base, in physiologically acceptable salt form, in form of lecithin, and/or in form of natural sources rich in lecithin or choline such as egg yolk, kidney, liver, heart, seeds, vegetables, and legumes, characterized in that choline is present in the formula diet in an amount of about 0.15 to 8 g per 100g of dry weight components (amount of choline in free base form). The formula diet will preferably further comprise other nutritionally advantageous components such as vitamins, minerals, trace elements, fibers (preferably soluble fibers).

Examples of suitable nitrogen sources include nutritionally acceptable proteins such as soy bean or whey derived proteins, caseinates, and/or protein hydrolysates. Suitable carbohydrate sources include sugars such as maltodextrins. Examples of suitable fat sources include triglycerides, as well as di- and monoglycerides.

Examples of vitamins suitable for incorporation into the medicament or nutritional formulation of the invention include Vitamin E, Vitamin A, Vitamin D, Vitamin K, folic acid, thiamin, riboflavin, Vitamin B₁, B₂, B₆ and B₁₂, niacin, biotin and pantothenic acid in nutritionally acceptable form.

Examples of mineral elements and trace elements suitable for incorporation into the medicament or nutritional formulation include sodium, potassium, calcium,

phosphorous, magnesium, manganese, copper, zinc, iron, selenium, chromium, and molybdenum in nutritionally acceptable form.

In particular, the medicament or nutritional formulation will preferably comprise beta-carotene (Vitamin A), Vitamin E, Vitamin C, Vitamin B₁, Vitamin B₁₂, selenium and zinc in nutritionally acceptable form.

The term "soluble fiber" as used herein refers to fibers which are able to undergo substantial fermentation in the colon ultimately to produce short chain fatty acids. Examples of suitable soluble fibers include pectin, guar gum, locust bean gum, xanthan gum which may optionally be hydrolysed. For adults, the total amount of soluble fibre per day will conveniently lie in the range of from 3 to 30g.

It will be appreciated that omega-3 PUFAs may be administered in higher amounts than those indicated hereinabove, and that such higher amounts will in general not impair the desired effect or provoke undesired side effects.

Compounds particularly suitable for use as component (d) in the nutritional formulation of the invention include L-arginine and L-ornithine, most preferably L-arginine. Component (d) may be employed in free form, physiologically acceptable salt form, e.g. in the form of a salt with phosphoric acid, citric acid, tartaric acid, fumaric acid, adipic acid or lactic acid, or in small peptide form. Preferably L-arginine in free form is employed.

The term small peptides as used herein refers to peptides having from 2 to 6, preferably from 2 to 4 amino acids.

As already indicated, omega-3 PUFAs will conveniently be administered in the form of fish oils, protected or not against peroxidation. Such fish oils also comprises omega-6 PUFAs.

Omega-6 PUFAs have also a favorable effect on the immune response and on the resistance to infection upon surgery. Accordingly, diets of the invention may conveniently further comprise omega-6 PUFAs.

For the purpose of the invention the omega-6 PUFAs may be in free acid form or in a form suitable for the physiological supply of omega-6 PUFAs, e.g. in triglyceride form. Examples of omega-6 PUFAs particularly appropriate for use according to the invention, include linoleic acid and arachidonic acid, linoleic acid being most preferred. Examples of suitable omega-6 PUFA sources are known in the art. They include fish oils and vegetable oils. Examples of omega-6 PUFA sources having a high linoleic acid content are safflower oil, sunflower oil, soy oil, cotton oil and corn oil.

Administration of a daily amount of omega-6 PUFAs in the range of from 1.5 to 5.0 g will in general suffice to attain a favorable effect. One unit dose of the medicaments or nutritional formulation defined above may accordingly further contain 1.5 to 5 parts by weight of omega-6 PUFAs.

In addition to components (b) to (e), and omega-6 PUFAs further components may be added to the diets of the invention and may have a beneficial effect on the activity of the glycine. An example of such beneficial components are omega-9 PUFAs. A preferred natural source for such fatty acid mixtures are fish oils. For taste and other reasons, the fish oils will, in oral application forms, preferably be used in encapsulated form.

Where the formula diet of the invention is intended for use as a complete formula diet which is part of the daily diet (e.g., pre-operative treatment), the amount of energy supplied by it should not be too excessive, in order not to unnecessarily suppress the patients appetite. The diet should conveniently comprise energy sources in an amount supplying from 600 to 1000 Kcal/day. For use as an exclusive complete formula diet (e.g., for post-operative treatment, treatment of trauma), the

diets of the invention will conveniently supply from 600 to 1500 Kcal/day. The contribution of the nitrogen source, carbohydrate source and lipid source to the total daily caloric supply may vary within wide ranges. In preferred formulations of the invention the carbohydrate source provides for 40 to 70 % of the total energy supply and, the nitrogen and fatty acid source each for 15 to 30 % of the total energy supply of the formulation. The formula diet will be either formulated in aqueous liquid form in volumes in the range of from 500 ml to 3000 ml or in powder form to be reconstituted in water prior to consumption.

Patients who can benefit from the present invention include patients undergoing major surgery, burn patients, patients suffering from acute trauma and/or sepsis, patients with preeclampsia, patients with acute liver disease caused by alcohol or with viral induced liver injury.

The nutritional formulations of the invention are as already set out above particularly suitable for treatment of patients due for surgery. Such pretreatment will be most effective when administering the formulations as complete formula diets taken as part of the daily diet. The nutritional formulations will advantageously be administered over a period of 2 days or longer. In general, a pretreatment starting 1 to 6 days before surgery, and during said 1-6 day period will be sufficient to attain the desired effect.

The nutritional formulations will conveniently be administered in the form of unit doses suitable for administration 2 to 4 times per day. Where the nutritional formulations comprise energy sources, it is appropriate not to supply more than 1500 Kcal/day. Apart from this limitation with respect to the energy supply, nutritional formulations of the invention can and will conveniently be supplied in the form of complete formula diets as described above.

Typical administration forms suitable for acute treatment are, e.g., the aqueous solutions disclosed hereinbelow.

Typical pharmacologically acceptable formulation forms for oral administration will further comprise pharmacologically acceptable diluents, carriers, vitamins, spices, pigments and/or other adjuvants well known to the skilled person to be suitable for incorporation into such formulation.

The diets and nutritional formulations of the invention may be obtained in a manner known per se, e.g., by admixing the ingredients.

Typical formulations suitable for use according to the invention containing choline and water include, e.g., aqueous solutions consisting essentially of 0.1 % to 90 % by weight of choline and 10% to 99.9% by weight of distilled water. Choline may be present in a concentrated form of the solution in an amount of from 15 to 90% (by weight of the solution). Concentrated solutions are suitable for dilution to application forms or for use in acute treatment. Application forms having a lower content (e.g. 0.1 to 5 %) of choline will in general be indicated for prophylactic purposes; concentrated forms of the solution having a higher content (e.g. 5 % to 40 % by weight) choline will in general be more suitable for acute treatment.

Other formulations suitable for inclusion in the medicament or formulation of the invention, in particular for parenteral application, include infusion solutions such as Ringer's injection solution, lactated Ringer's injection solution, crystalloids, colloids or other plasma substitutes, in association or enriched with about 0.15 to 15 g, preferably 4 to 12 g per liter infusion solution of choline. Ringer's injection solution is a sterile solution, containing from 3.23 to 3.54g of sodium (equivalent to from 8.2 to 9.0g of sodium chloride), from 0.149 to 0.165 of potassium (equivalent to from 0.285 to 0.315g of potassium chloride), from 0.082 to 0.098g of calcium (equivalent to from 0.3 to 0.36g of calcium chloride, in the form of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), from 5.23 to 5.80g of chloride (as NaCl , KCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and water in sufficient quantity to give 1000 ml solution. Lactated Ringer's Injection solution is a sterile solution containing from 2.85g to 3.15g sodium, as chloride and lactate),

from 0.141 to 0.173g of potassium (equivalent to from 0.27g to 0.33g of potassium chloride), from 0.049 to 0.060g calcium (equivalent to from 0.18g to 0.22g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), from 2.31g to 2.61g of lactate, from 3.68 to 4.08g of chloride (as NaCl , KCl and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) and water in sufficient quantity to give 1000 ml solution.

The terms crystalloids and colloids in connection with fluid therapy are known in the art. They include plasma substitutes such as HaemaccelTM (polygeline based) and GelofusineTM (gelatin based).

The invention will be further apparent from the following description of the figures and the associated figures.

Figure 1 shows dose-response of dietary choline on survival after LPS. Rats were fed chow with various amounts of added choline chloride for 3 days. LPS (5 mg/kg) was injected via the tail vein and survival was assessed after 24 h. Fractions represent survivors/total.

Figure 2 shows the effect of choline on LPS-induced mortality. Rats were fed chow or chow + 0.4% choline for 3 days. LPS was injected into the tail vein and mortality was assessed after 24 h. Fractions presented are survivors/total. *, p<0.05; Fisher's exact test.

Figure 3 shows the effect of choline on LPS-stimulated serum AST (aspartate aminotransferase). Conditions as in Fig. 1. Blood samples were collected 8 h after injection of 5 mg/kg LPS. *, p<0.05 compared to choline control, *, p<0.05 compared to chow + LPS by Mann-Whitney's rank-sum test; n=4.

Figure 4 shows the effect of choline on LPS-stimulated intracellular calcium levels in isolated Kupffer cell. $[\text{Ca}^{2+}]_i$ in cultured Kupffer cells from chow (A) or chow + choline-fed rats (B) was measured fluorometrically using fura-2. Addition of LPS (10 $\mu\text{g}/\text{ml}$) is denoted with horizontal bars and arrows. Representative traces.

Figure 5 shows the effect of choline on peak intracellular calcium levels following addition of LPS to isolated Kupffer cells. Conditions as in Fig. 4. Mean \pm SEM of peak $[Ca^{2+}]_i$ following LPS. ***, p<0.001 using student's *t*-test.

Figure 6 shows the effect of choline on serum TNF- α levels after LPS injection. Blood samples were collected before and 1 h following injection of 5 mg/kg LPS. TNF- α was measured by enzyme-linked immunosorbent assay as detailed in METHODS. *, p<0.05 using student's *t*-test; n=6.

Figure 7 shows the effect of choline on serum triglyceride levels. Blood samples were taken 8 h after LPS (5mg/kg). Triglycerides were measured enzymatically in serum. *, p<0.05 by one-way analysis of variance; n=4.

The invention will be further understood by reference to the following specific description of the examples.

EXAMPLES

Methods

Dietary treatment. Female Sprague-Dawley rats (250-275 g) were fed standard laboratory chow (Agway PROLAB RMH 3000, Syracuse, NY), chow and choline chloride (0.05-0.4%), chow and glycine (5%), or chow and 5% glycine and 0.4% choline chloride for 3 days. Rats were given free access to water and were maintained on a 12 h light/dark cycle.

Endotoxin treatment. After 3 days of feeding the rats the diets described above, LPS (*Escherichia coli* serotype 0111:b4; Sigma Chemical, St. Louis, MO) was suspended in pyrogen-free saline and injected into the tail vein. Survival was assessed after 24 h, and in some experiments, blood and tissue samples were collected 8 h after LPS injection. Serum was stored at -20°C for measurement of total triglycerides and

aspartate aminotransferase activity by standard enzymatic methods (Bergmeyer HU. Methods of Enzymatic Analysis. New York: Academic Press, 1988 and Bucolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 1973; 19:476-482.). Liver and lung samples were fixed in phosphate-buffered formalin and embedded in paraffin. Blind evaluation of hematoxylin and eosin stained sections was performed.

TNF- α measurement. LPS (5mg/kg) was injected via tail vein. After 1h, blood samples were collected from the vena cava to determine circulating levels of TNF- α . To each 200 μ l blood sample, 75 μ l of aprotinin (Sigma) was added and TNF- α was measured by enzyme-linked immunosorbent assay (ELISA; Genzyme, Cambridge, MA). Livers were removed and immediately frozen in liquid nitrogen for measurement of TNF- α mRNA. Reverse transcriptase polymerase chain reaction and densitometry were used to quantify mRNA.

Kupffer cell isolation and culture. Kupffer cells were isolated from rats fed chow or choline-supplemented diets by collagenase digestion and differential centrifugation as described previously (Pertoft H. and Smedsrød B. Separation and characterization of liver cells. Pretlow II TG, Pretlow TP. Cell Separation: Methods and Selected Applications, Vol. 4, ed. Academic Press, 1987:1-24). Briefly, the portal vein was cannulated and livers were perfused with calcium and magnesium-free Hanks' balanced salt solution (HBSS; 37°C) for 5 min. Perfusion was continued with HBSS containing 0.025% collagenase type IV (Sigma) for approximately 5 min. When digestion appeared complete, the liver was removed, placed in a beaker containing collagenase buffer and cut into small pieces. The suspension was filtered through nylon gauze and centrifuged for 10 min at 450 x g at a temperature of 4°C. The cell pellet was resuspended in HBSS and parenchymal cells were removed by centrifugation at 50x g for 3 min. The nonparenchymal cell fraction was washed twice with buffer. Kupffer cells were isolated by centrifugation on a Percoll cushion at 1000 x g for 15 min. Viability was determined by trypan blue exclusion and was >90%. Cells were seeded on glass coverslips and culture medium was exchanged after 1 h to remove nonadherent cells. The culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G and 100

µg/ml streptomycin sulfate. Purity was determined from the percentage of cells that engulfed latex beads by phagocytosis and was found to be near 100%. Cells were cultured for approximately 24 h prior to experiments.

Measurement of [Ca²⁺]_i. The fluorescent Ca²⁺ indicator dye fura-2 was used to measure intracellular Ca²⁺ as detailed previously (Ikejima K, Thurman RG. Glycine prevents elevation in intracellular Ca²⁺ in Kupffer cells by endotoxin (LPS) via actions on a chloride channel. *Hepatology* 1995; 22:803). Briefly, Kupffer cells plated on coverslips were incubated in modified Hanks' buffer and 0.03% Pluronic F-127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 1 h. Changes in fluorescence intensity of fura-2 at excitation (340 and 380 nm) and emission (520 nm) wavelengths was monitored in individual Kupffer cells and values were corrected for system noise and autofluorescence.

Statistical analysis. Results are represented as mean \pm SE. Significance was determined using Student's t-test or Mann-Whitney's rank-sum where appropriate. The Fisher exact test was used to determine significance in the mortality studies. p<0.05 was selected as the level of significance.

Results

Effect of choline on survival after LPS injection. There were no significant differences in the average daily consumption or chow or choline-supplemented diets (18.1 \pm 1.3 and 18.5 \pm 1.3 g, respectively). Body weight for the two groups were also similar (chow 364 \pm 4g; choline 365 \pm 3g). After three days of feeding the rats specialized diets, LPS (2.5-20mg/kg) was injected via the tail vein. Seventeen percent of rats fed chow died within 24 h following 2.5 mg/kg LPS, 56% died at the 5 mg/kg dose, 83% at 10 mg/kg, and 100% at 20 mg/kg (Fig. 1). Death usually occurred 8-12 h after LPS and surviving animals showed improvement after 24 h. The addition of 0.4% choline to the diet increased survival to 100% after injection of 2.5 or 5.0 mg/kg LPS (p<0.05) while 44% of rats survived at the 10 mg/kg dose (Fig. 1). Choline did not increase survival after 20 mg/kg LPS. The effect of increasing the amount of choline in the diet was also observed. Survival after a sublethal dose of LPS (5mg/kg) increased from 17% (no addition) to 100% and 75% with the addition of 0.05% and 0.1%

choline, respectively as shown in Fig. 2. Dietary supplementation with 0.4% choline significantly improved survival to 100% (p<0.05).

Glycine and choline are additive. In a previous study, dietary supplementation with glycine blunted Kupffer cell activation and increased survival due to endotoxin shock (Ikejima K, Iimuro Y, Forman DT, Thurman RG. A diet containing glycine improves survival in endotoxin shock in the rat. Am J Physiol 1996, 271:G97-G103). Glycine most likely inhibits Kupffer cell activation due to LPS by hyperpolarization of the plasma membrane by activating a glycine-gated chloride channel. To determine if the effects of glycine and choline are additive, rats fed chow, chow supplemented with glycine or choline, or a combination of both were given LPS (10mg/kg) as described above. Under these conditions 17% of chow-fed animals and 43% of choline-fed animals survived (Table 1). Glycine alone improved survival by 50%; however, given together, glycine and choline increased survival to 100%. Since the effects of glycine and choline are additive in this model, it is likely that choline acts at a site distinct from the choline channel affected by glycine.

Table 1. *Effect of feeding diets containing glycine and choline on LPS-induced mortality.*

	<u>Survivors/Total</u>	<u>% Survivors</u>
No Addition	1/6	17
0.4% Choline	3/7	43
5% Glycine	4/6	67
5% Glycine +	6/6	100*
0.4% Choline		

Rats were fed a chow-based diet supplemented with 5% glycine or 0.4% choline chloride. Survival was assessed 24 h after injection of 10 mg/kg LPS as described above (n=6). *p<0.05; Fisher's exact test.

Effect of choline on serum AST and histology after LPS. Blood samples were collected 8 h after injection of 5 mg/kg LPS. Basal serum AST (aspartate aminotransferase) values were 65 \pm 33 U/l and 55 \pm 18 U/l in the chow and chow + 0.4% choline groups, respectively (Fig. 3). Injection of LPS increased AST to 846 \pm 146 U/l in chow fed animals; however, this increase was significantly blunted by feeding choline with values only reaching 163 \pm 22 U/l (p<0.05); Mann-Whitney rank sum test). LPS induced massive necrosis and inflammation in livers from chow-fed rats. Pulmonary edema and marked increase in infiltrating inflammatory cells was also observed. Liver necrosis and pulmonary edema were both attenuated in choline-treated rats; choline significantly diminished liver and lung pathology scores.

Effect of choline on changes in [Ca²⁺]_i in cultured Kupffer cells. Intracellular calcium was monitored fluorometrically in Kupffer cells isolated from chow or choline-fed animals (Figs. 4 & 5). After the addition of 10 μ g/ml LPS to the culture medium of Kupffer cells isolated from chow-fed rats, [Ca²⁺]_i rapidly increased reaching a peak value within 100 sec (241 \pm 17 nM; n=4), and declined to basal levels within 200 sec. The addition of 10 mM choline chloride to the culture medium 6 or 24h prior to LPS did not affect increases in calcium caused by LPS (data not shown). However, when Kupffer cells were isolated from rats fed 0.4% choline for three days, the increase in [Ca²⁺]_i due to LPS was only approximately 50% as large (Figs. 4&5).

Effect of choline on serum TNF- α levels. Peak TNF- α release occurs approximately 1 h after injection of LPS. Therefore, in the present study serum TNF- α was measured 1 h after injection of 5mg/kg LPS. There was an increase in serum TNF- α (69 \pm 6 ng/ml). The increase serum TNF- α was blunted by choline (58 \pm 8 ng/ml; p<0.08 by Student's t test). Endotoxin activates macrophages that, in turn, produce cytokines that mediate the host response to injury. TNF- α is believed to be responsible for the increases in triglyceride synthesis as well as increased production of VLDL during infection. Since protective effects of choline correlated with lower TNF- α production in response to LPS this indicated that the mechanism of choline protection most likely involves diminished macrophage activation.

Effect of choline on serum triglyceride levels. Gram-negative bacterial sepsis and endotoxin shock cause a disturbance in lipid metabolism that leads to hypertriglyceridemia, characterized by increased VLDL secretion. It has been shown that lipoproteins bind and detoxify endotoxin and treatment with lipoproteins such as VLDL protects against endotoxin-induced mortality. Therefore, the increase in lipoprotein production due to endotoxin may be a component of the defence mechanism against infection. Since choline is necessary for the synthesis of VLDL it was hypothesized by the present inventors that choline would increase survival by increasing circulating levels of very low density lipoprotein (VLDL), a lipoprotein that binds and detoxifies endotoxin. Since triglycerides compose 60% of VLDL, serum triglycerides were measured as a marker of VLDL. Prior to injection of LPS, serum triglyceride levels were within the normal range in chow and chow + choline rats (177 ± 9 and 160 ± 7 mg/dl, respectively). Following LPS injection triglyceride levels increased to 333 ± 51 mg/dl in chow-fed rats, an effect that was surprisingly blocked by choline (165 ± 16 mg/dl) rather than enhanced. Therefore, it is unlikely that the mechanism of choline protection involves VLDL.

Liquid Enteral Compositions

Typically 1500ml of these liquid enteral compositions will be administered per 24 h. In the following compositions MM stands for "mineral mixture", SM for "trace element mixture" and VM for "vitamin mixture". The composition of these three mixtures is as follows:

<u>MM</u>		<u>VM</u>	
<u>Ingredients</u>	<u>g/100g</u>	<u>Ingredients</u>	<u>g/100g</u>
Maltodextrins	34.40	Maltodextrins	43.44
Potassium citrate/phosphate	34.60	Sodium ascorbate	35.00
Magnesium dicitrate	8.20	Vitamin E-Ac. 50%	16.00
Calcium chloride	8.00	Niacinamide	1.55
Sodium citrate/chloride	9.00	Vitamin A-Acetate	1.20
Citric acid	3.50	Ca-D-Panthenate	0.98
Choline tartrate	2.30	Vitamin K ₁ 1%	0.71
		Vitamin B ₁₂ 0.1%	0.30
		Vitamin D ₃	0.28
		Vitamin B ₆	0.20
		Vitamin B ₁	0.17
<u>SM</u>		Vitamin B ₂	0.15
<u>Ingredients</u>	<u>g/100g</u>	<u>Ingredients</u>	<u>g/100g</u>
Maltodextrins	52.54	Folic acid	0.02
Na-molybdate 1% Mo	17.30	Biotin	0.01
Chromium-III-Cl 1% Cr	8.80		
Zinc sulfate	7.00		
Na-selenite 1% Se	3.35		
Ferrum(II) sulfate	6.92		
Copper(II) gluconate	2.24		
Manganese(II) sulfate	1.12		
Sodium fluoride	0.70		
Potassium iodide	0.03		

Composition Comprising Choline

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	11.95

Na/Ca caseinates	4.60
Choline tartrate	1.15
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	2.33
Sunflower oil	0.26
Emulsifier Nathin E	<u>0.13</u>
	100.00

Composition Comprising Choline and Glycine

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	8.95
Na/Ca caseinates	4.60
Choline tartrate	1.15
Glycine	3.00
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	2.36
Sunflower oil	0.23
Emulsifier Nathin E	<u>0.13</u>
	100.00

Composition Comprising Choline and Fish Oil (ω-3 fatty acids)

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	11.95
Na/Ca caseinates	4.60
Choline tartrate	1.15
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	1.32
Sunflower oil	0.23
Emulsifier Nathin E	0.13
Fish Oil EPAX 3000 TG	<u>1.04</u>
	100.00

Composition Comprising Choline and Arginine

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	10.78
Na/Ca caseinates	4.60
Choline tartrate	1.15
Arginine	1.17
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03

Palm oil	2.33
Sunflower oil	0.26
Emulsifier Nathin E	<u>0.13</u>
	100.00

Composition Comprising Choline, Glycine and Fish Oil (ω-3 fatty acids)

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	8.95
Na/Ca caseinates	4.60
Choline tartrate	1.15
Glycine	3.00
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	1.32
Sunflower oil	0.23
Emulsifier Nathin E	0.13
Fish Oil EPAX 3000 TG	<u>1.04</u>
	100.00

Composition Comprising Choline, Glycine and Arginine

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	7.78
Na/Ca caseinates	4.60
Choline tartrate	1.15
Glycine	3.00

Arginine	1.17
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	2.33
Sunflower oil	0.26
Emulsifier Nathin E	<u>0.13</u>
	100.00

Composition Comprising Choline, Glycine, Arginine and Fish Oil (ω-3 fatty acids)

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	7.80
Na/Ca caseinates	4.60
Choline tartrate	1.15
Glycine	3.00
Arginine	1.17
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	1.32
Sunflower oil	0.23
Emulsifier Nathin E	0.13
Fish Oil EPAX 3000 TG	<u>1.04</u>
	100.00

Composition Comprising Choline, Glycine, Arginine, RNA and Fish Oil (ω-3 fatty acids)

<u>Ingredients</u>	<u>g/100g</u>
Water	77.40
Maltodextrins	7.67
Na/Ca caseinates	4.60
Choline tartrate	1.15
Glycine	3.00
Arginine	1.17
Yeast extract RNA	0.14
MM	2.00
SM	0.05
VM	0.10
β-Carotine	0.03
Lipids:	
Palm oil	1.32
Sunflower oil	0.23
Emulsifier Nathin E	0.13
Fish Oil EPAX 3000 TG	<u>1.04</u>
	100.00

As already set out above, fish oil is a natural source for omega-3 PUFAs (contains about 30% omega-3 PUFAs whereas sunflower oil is a natural source for omega-6 PUFAs. About 47% by weight of choline tartrate is choline in free base form.

Composition in Powder Form for Oral Administration

The following composition is designed as an oral complete formula diet to be taken as part of the daily diet. Typical daily dosages will range from 148 to 222 g of powder to be reconstituted in water prior to oral consumption (corresponds roughly to 600 to 900 kcal per day).

<u>Ingredients</u>	<u>g/100g</u>
Whey protein	23.000000
Sucrose	26.398000
Choline-H-tartrate	13.040000
Glycine	11.425000
L-Arginine	5.100000
Fish oil	4.600000
Hydrolysed soluble fibres	4.500000
MCT oil	3.000000
Corn oil	2.000000
Na-chloride	0.900000
Yeast extract RNA	0.620000
Calciumphosphate, tri	0.600000
Mg-dicitrate	0.580000
K-citrate	0.540000
Citric acid	0.540000
Lemon aroma	0.500000
Orange aroma	0.500000
Emulsifier Nathin E	0.440000
Stab. Keltrol F	0.400000
K-H-phosphate, di	0.324000
Maltodextrin 10 DE	0.210905
Na-ascorbate, cryst.	0.155000
Passion Fruit aroma	0.150000
Colour beetroot	0.150000
Colour B-carotene 1%	0.118000
Vitamin E-Ac.50%	0.069000
Antiox Ronoxy A	0.060000
Zinc-sulfate 1-hydr	0.016800

<u>Ingredients</u>	<u>g/100g</u>
Fe-II-sulfate	0.016608
Na-molybdate 1% Mo	0.008640
Nicotinamide	0.006400
Cu-II-gluconate	0.005376
Vit. A-acetate	0.005040
Ca-D-pantothenate	0.005000
Chromium-III-Cl 1% Cr	0.004416
Mn-II-sulfate 1-hydr	0.002688
Vitamine K1 1%	0.002500
Na-selenite 1% Se	0.001680
Na-fluoride	0.001680
Vitamine B6-HCl	0.000840
Vitamin B2	0.000750
Vitamin B1-HCl	0.000750
Vitamin D3	0.000700
Folic Acid	0.000100
Potassium jodide	0.000082
<u>Biotin, d</u>	<u>0.000045</u>
Sum of Ingredients	100.000000

CLAIMS

1. The use of choline in the preparation of a medicament or nutritional formulation for the reduction and/or prevention of endotoxin-induced injury and/or mortality.
2. The use according to claim 1 wherein a daily dosage form comprises 1.5 to 20 grams of choline.
3. The use according to claim 1 or 2 wherein the medicament or nutritional formulation further comprises glycine.
4. The use according to claim 3 wherein a daily dosage form comprises 1.5 to 20g of choline and 1.5 to 75 grams of glycine.
5. Use of choline in the preparation of a medicament or nutritional formulation for the treatment of endotoxin-related injury.
6. The use of claim 5 wherein the endotoxin-related injury is alcohol-induced liver injury.
7. The use of any one of the previous claims wherein the choline is administered enterally.
8. A nutritional composition comprising, as a daily dosage form, from 1.5 to 20 g of choline and a nutritionally acceptable carrier.
9. A nutritional composition comprising:
1.5 to 20 parts of choline,
1.5 to 75 parts of glycine, and
a nutritionally acceptable carrier,

wherein in a daily dosage form the composition provides from 1.5 to 20 g of choline and from 1.5 to 75 g of glycine.

10. A composition comprising

- (a) choline, in association with one or more components selected from the group consisting of:
- (b) glycine,
- (c) omega-3 fatty acids,
- (d) L-arginine or other physiologically acceptable compounds associated with the synthesis of polyamines, or mixtures thereof, and
- (e) a nucleobase source,

in an effective amount collectively to reduce endotoxin-induced injury or mortality, or both, and a nutritionally acceptable carrier.

11. The composition according to claim 10 comprising in parts by weight,

- (a) 1.5 to 20 parts choline, in association with one or more components selected from the group consisting of:
- (b) 1.5 to 75 parts glycine,
- (c) 0.1 to 20 parts omega-3 fatty acids;
- (d) 3 to 40 parts L-arginine or other physiologically acceptable compounds associated with the synthesis of polyamines, or mixtures thereof, and
- (e) 0.1 to 4 parts nucleobase source,

and a nutritionally acceptable carrier.

12. The composition according to claim 11 comprising in parts by weight as a unit dose,

- (a) 1.5 to 20 parts choline, in association with one or more components selected from the group consisting of:
- (b) 2 to 60 parts of glycine,
- (c) 2 to 5 parts omega-3 fatty acids,

(d) 7.5 to 20 parts L-arginine or other physiologically acceptable compounds associated with the synthesis of polyamines, or mixtures thereof, and

(e) 1.7 to 2 parts nucleobase source,
and a nutritionally acceptable carrier.

13. A nutritional composition comprising a carbohydrate source, a fat source, a nitrogen source, and choline, as a free base, in physiologically acceptable salt form or in form of lecithin wherein the choline is added in a weight amount of the free base form of from about 1.5 to 8 g per 100g of dry weight components of the composition.

14. The composition according to claim 14 further comprising one or more of vitamins, minerals, trace elements and fibers.

15. The composition according to any one of claims 8 to 14 in a form suitable for enteral administration.

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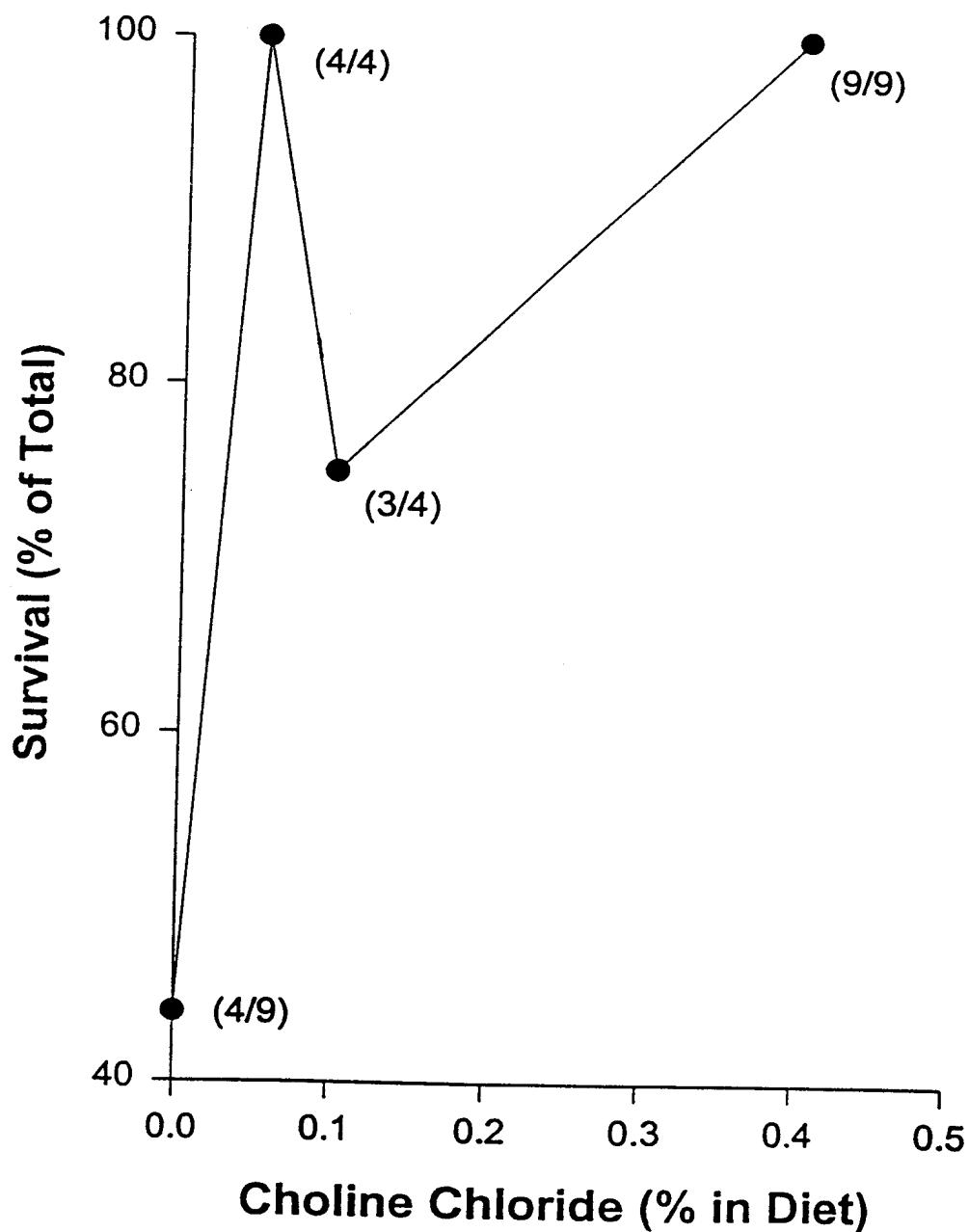


Fig. 1

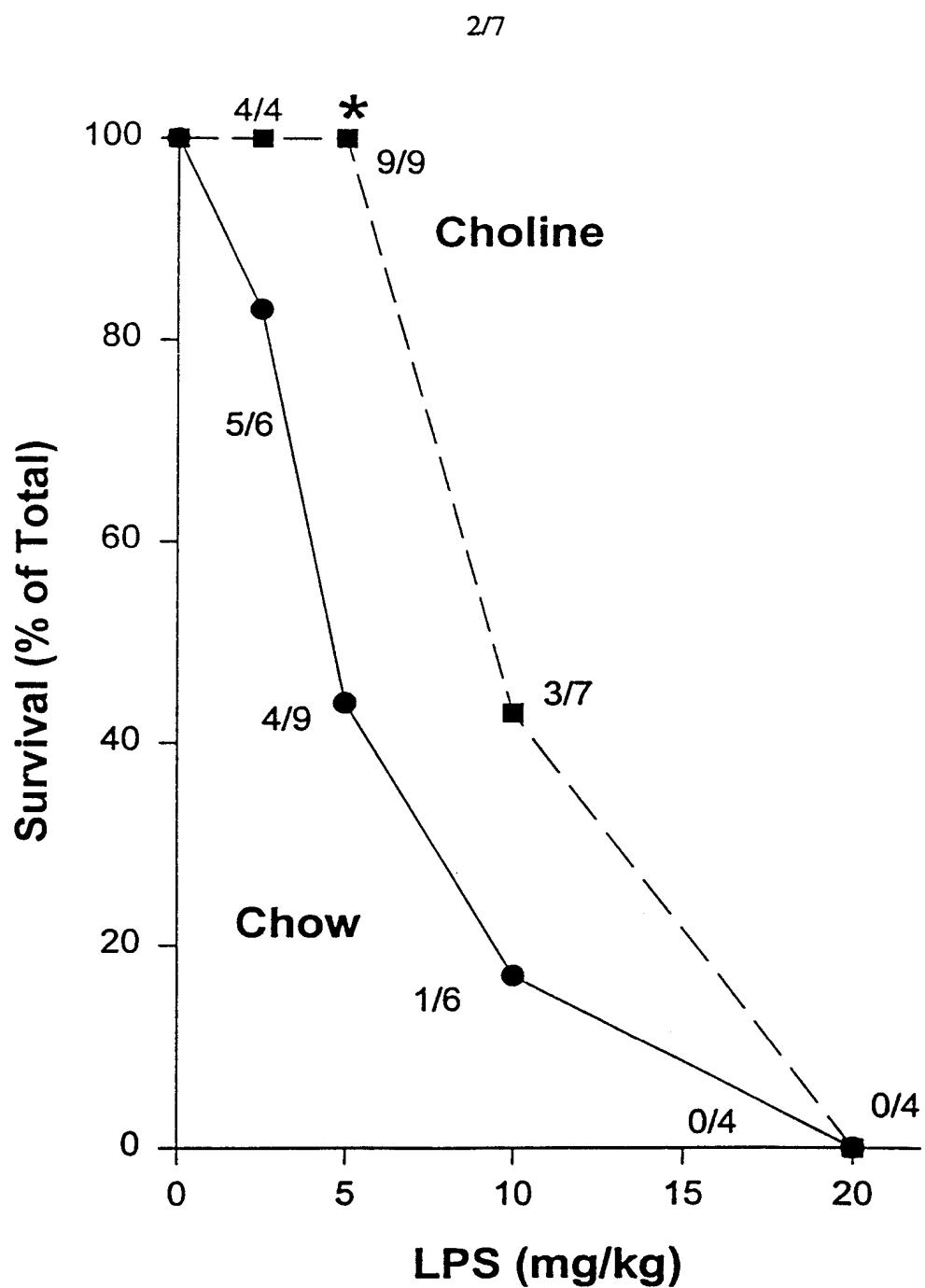


Fig. 2

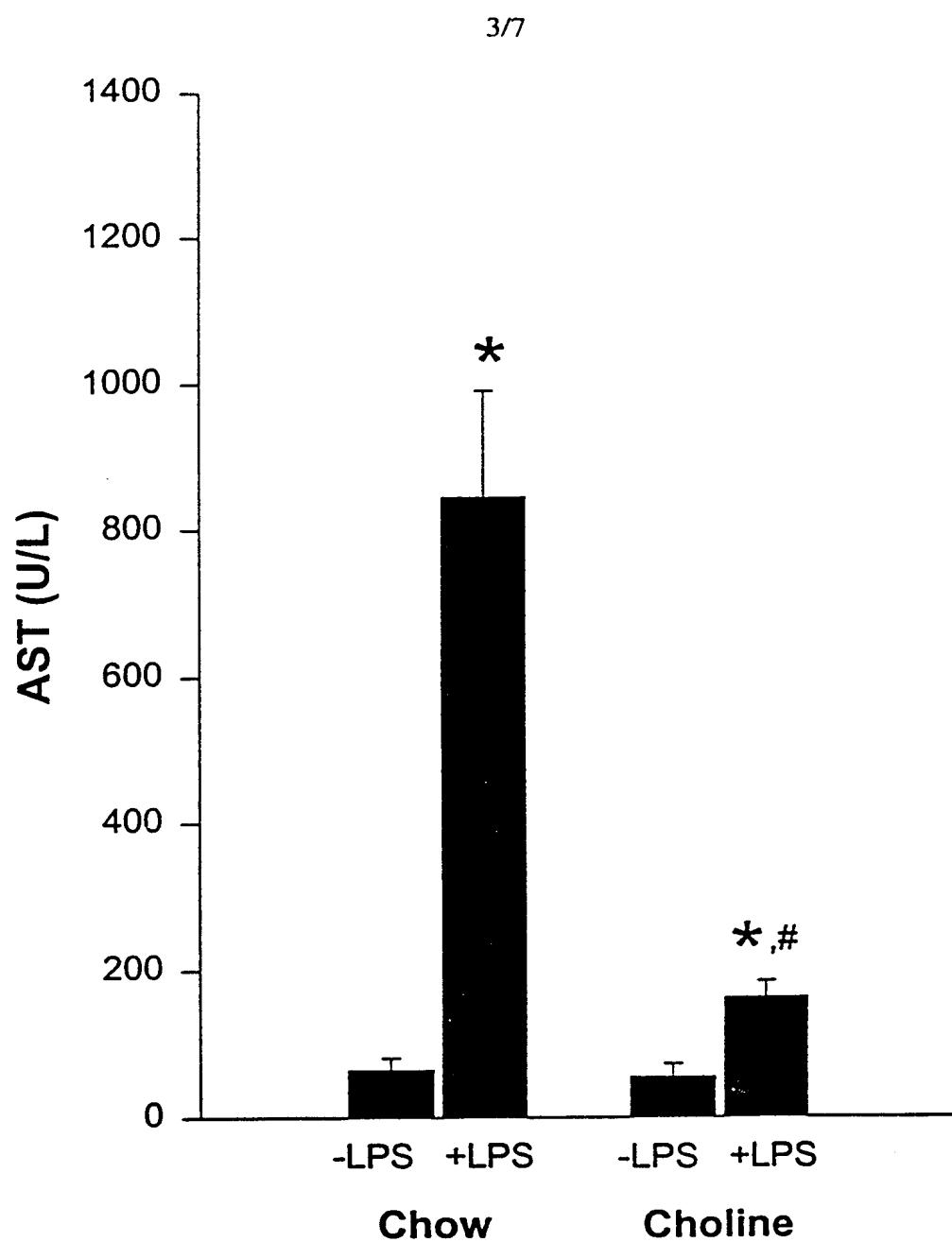


Fig. 3

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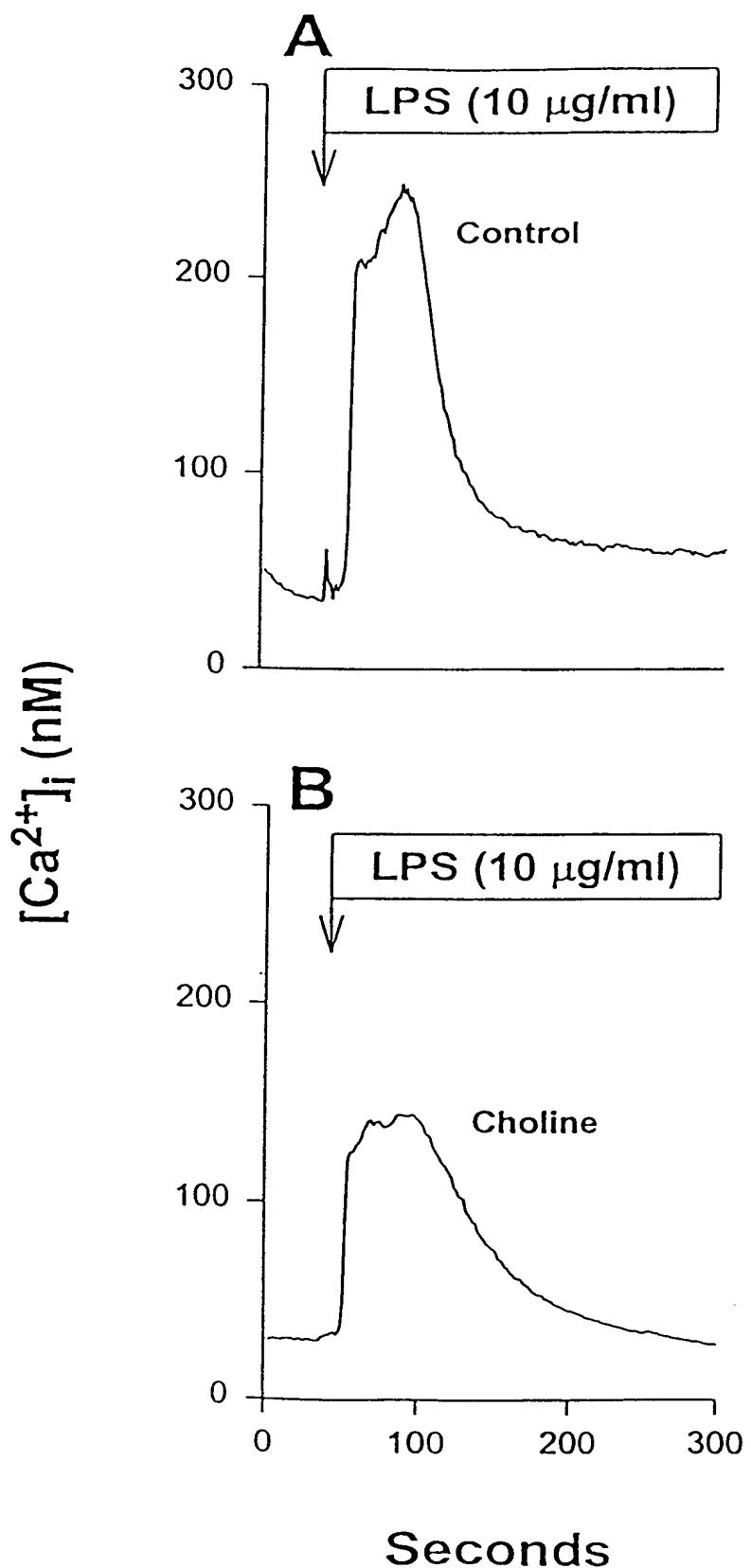


Fig. 4
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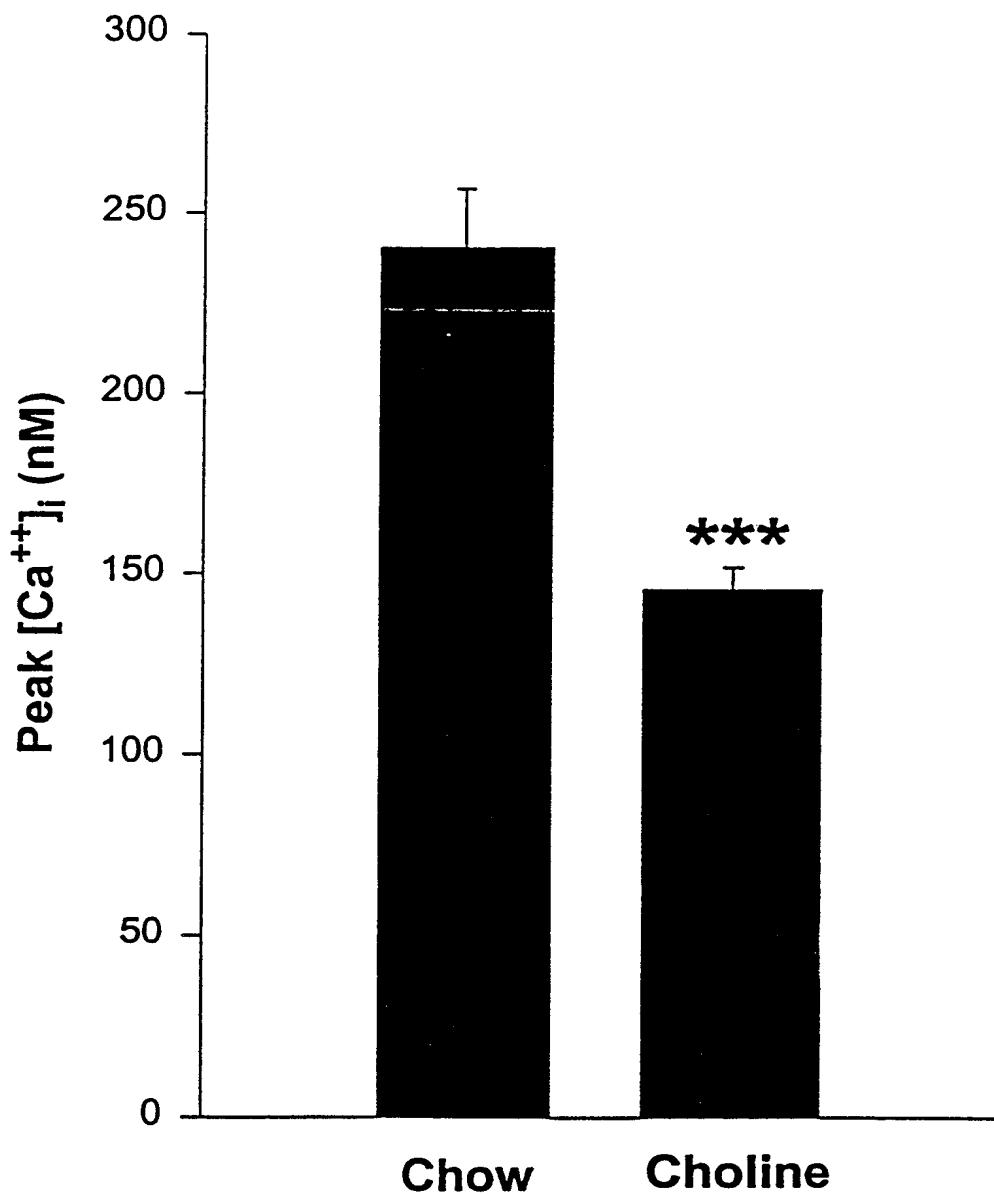


Fig. 5

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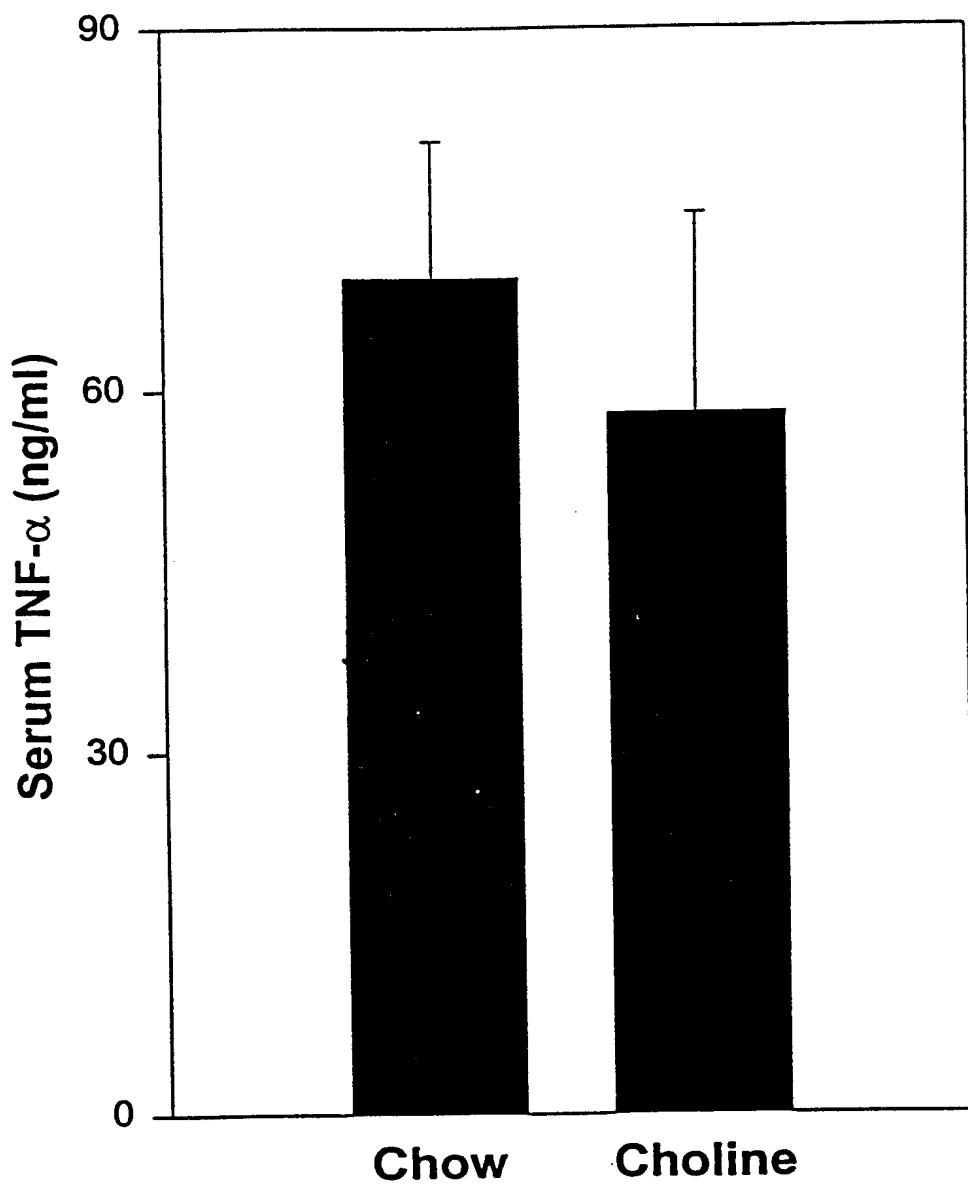


Fig. 6

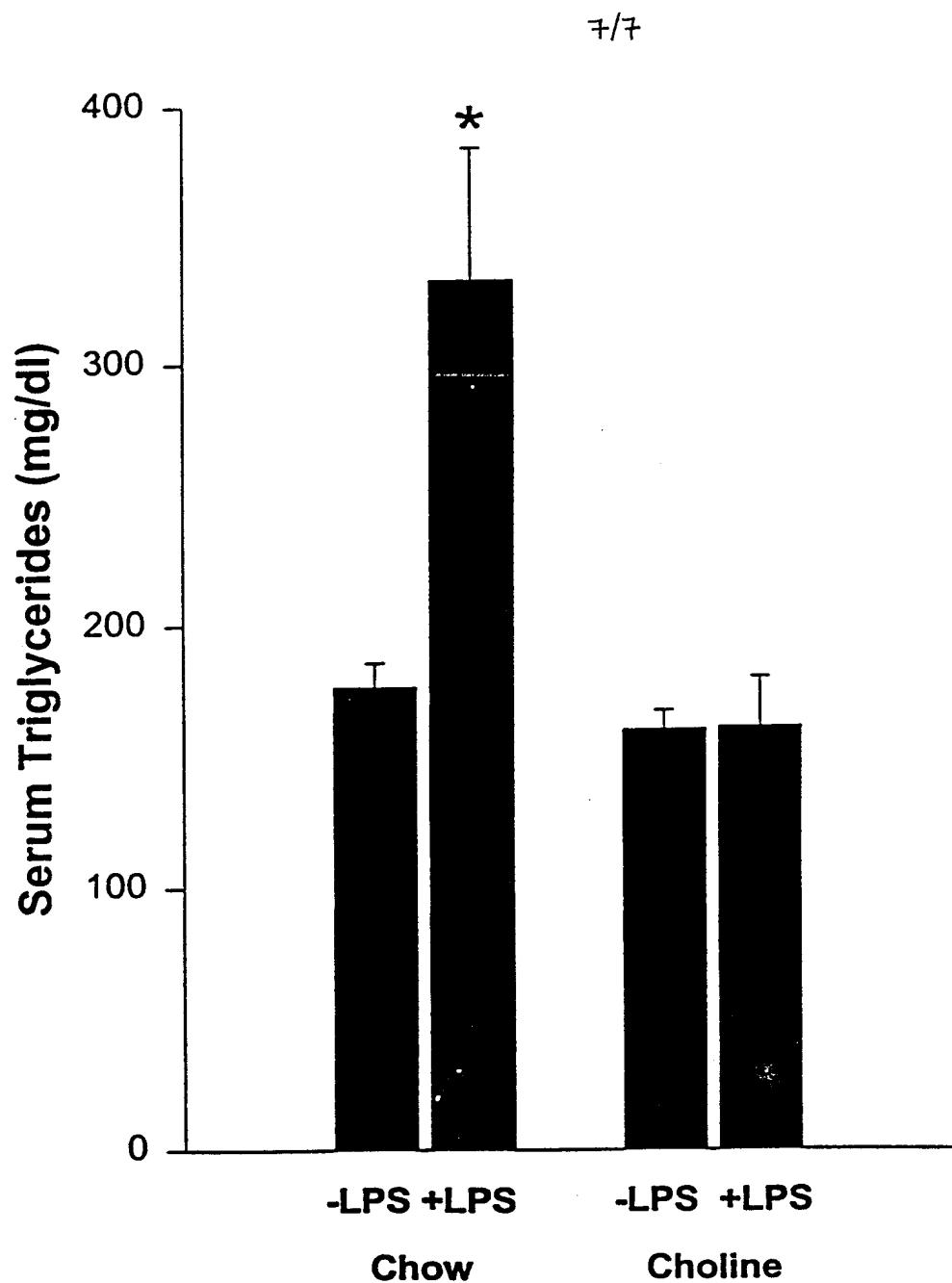


Fig. 7

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/14, 31/66		A3	(11) International Publication Number: WO 98/32428																			
			(43) International Publication Date: 30 July 1998 (30.07.98)																			
<p>(21) International Application Number: PCT/EP98/00373</p> <p>(22) International Filing Date: 23 January 1998 (23.01.98)</p> <p>(30) Priority Data: 08/789,773 27 January 1997 (27.01.97) US</p> <p>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/789,773 (CIP) Filed on 27 January 1997 (27.01.97)</p> <p>(71) Applicant (for all designated States except US): NOVARTIS NUTRITION AG [CH/CH]; Monbijoustrasse 118, CH-3001 Bern (CH).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): SCHNEIDER, Heinz [CH/CH]; Buillard, CH-1792 Cordast (CH). THURMAN, Ronald, G. [US/US]; 810 Mt. Creek Road, Chapel Hill, NC 27516 (US).</p> <p>(74) Agent: SMOLDERS, Walter; Novartis AG, Patent- und Markenabteilung, Lichtstrasse 35, CH-4002 Basel (CH).</p>			<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p> <p>(88) Date of publication of the international search report: 12 November 1998 (12.11.98)</p>																			
<p>(54) Title: COMPOSITIONS COMPRISING CHOLINE AND USE OF CHOLINE TO TREAT ENDOTOXIC SHOCK</p> <p>(57) Abstract</p> <p>The invention provides a method for the treatment of endotoxic shock comprising administering to a human or other mammal in need of such a treatment an effective amount of choline for reducing endotoxin-induced injury and mortality and a nutritional composition comprising choline, whereby the composition provides, in a daily dosage form, from 1.5 to 20 g of choline.</p> <table border="1"> <caption>Data from Figure: Survival (% of Total) vs LPS (mg/kg)</caption> <thead> <tr> <th>LPS (mg/kg)</th> <th>Choline Survival (% of Total)</th> <th>Chow Survival (% of Total)</th> </tr> </thead> <tbody> <tr> <td>0</td> <td>100</td> <td>100</td> </tr> <tr> <td>2</td> <td>82 (5/6)</td> <td>44 (4/9)</td> </tr> <tr> <td>5</td> <td>44 (4/9)</td> <td>16 (1/6)</td> </tr> <tr> <td>10</td> <td>42 (3/7)</td> <td>16 (1/6)</td> </tr> <tr> <td>20</td> <td>0 (0/4)</td> <td>0 (0/4)</td> </tr> </tbody> </table>					LPS (mg/kg)	Choline Survival (% of Total)	Chow Survival (% of Total)	0	100	100	2	82 (5/6)	44 (4/9)	5	44 (4/9)	16 (1/6)	10	42 (3/7)	16 (1/6)	20	0 (0/4)	0 (0/4)
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10	42 (3/7)	16 (1/6)																				
20	0 (0/4)	0 (0/4)																				

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/00373

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/14 A61K31/66

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J.E.F. REYNOLDS, ED.: "MARTINDALE The Extra Pharmacopoeia" 1996, ROYAL PHARMACEUTICAL SOCIETY, LONDON XP002073856 see page 1356 - page 1357 ---	1-15
X	P.H.LIST ET AL.: "HAGERS HANDBUCH DER PHARMAZEUTISCHEN PRAXIS, 4th D., Volume 3" 1972, SPRINGER-VERLAG, BERLIN - HEIDELBERG - NEW YORK XP002073857 see page 885 - page 890 ---	1-15
X	FR 874 M (BIOREX LABORATORIES LIMITED) 16 October 1961 see the whole document ---	1-15 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

Date of mailing of the international search report

6 August 1998

20/08/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Theuns, H

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/EP 98/00373

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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